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MATHEMATICAL METHODS FOR CELL CYCLE ANALYSIS FROM FLOW CYTOMETRIC DNA-BrdUrd DISTRIBUTIONS

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The paper discusses some mathematical methods, presented in the literature or proposed by the authors, which aim at the estimation of cell kinetic parameters from DNA-BrdUrd distributions obtained by the pulse labelling technique. In particular, we give an expression for the time behaviour of the relative movement of BrdUrd-labelled undivided cells, that takes into account the variability of DNA synthesis rate across S phase.

1. Introduction

An experimental approach currently used for the analysis of the cell cycle is based on recognizing cells that synthesize DNA by immunofluorescence, using an antibody specific for bromodeoxyuridine (see e.g. Dean *et al.*, 1984). In the pulse labelling technique, cells are incubated in culture medium containing bromodeoxyuridine (BrdUrd) for a short time interval (15–60 min). BrdUrd, an analogue of thymidine, is incorporated into the DNA of DNA-synthesizing cells. Immediately or at a given time after the incorporation the cells are fixed and, after partial DNA denaturation, reacted with anti-BrdUrd monoclonal antibodies which are either labelled with fluorescein isothiocyanate (that emits green fluorescence), or successively recognized by another fluorescein-labelled antibody. Then DNA is stained by a DNA-specific dye, typically propidium iodide, that emits red fluorescence. A sample of the dual-stained cells is finally processed by a flow cytometer. The measurements of green and red fluorescences of each cell are accumulated to form a bivariate fluorescence histogram.

The DNA-BrdUrd histogram measured at the end of the pulse usually allows a straightforward recognition of $G1$, S, and $G2M$ cells, since cells in active DNA synthesis emit high levels of green fluorescence (see Fig. 1). Thus, the phase fractions can be estimated by counting the cells within suitable regions in the red-green fluorescence plane (Dean *et al.*, 1984). The close association between BrdUrd incorporation and DNA synthesis suggests that DNA-BrdUrd fluorescence histograms can give information on the rate of DNA synthesis. It has been pointed out (Dean *et al.*, 1984; Sasaki *et al.*, 1986) that the profile of the mean green fluorescence across

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of $G1$ peak equal to 3–4%) the deformation due to the finite pulse length is localized at the boundaries of S phase.

We stress finally that the linearity assumptions iii) and iv), crucial for our analysis, require a careful evaluation of the experimental protocol. For instance, it has been shown (Hoy *et al.*, 1989) that the linearity between BrdUrd-fluorescence and incorporated BrdUrd can be lost at the step of DNA denaturation, with a consequent additional deformation of the histogram shape.

3. The Relative Movement Method

The DNA-BrdUrd distributions measured at increasing times after the pulse show a progressive displacement of the BrdUrd-positive cells across the S phase. Begg *et al.* (1985) defined as relative movement (of the labelled undivided cells), RM, the quantity

$$RM = \frac{F^{lu} - F_{G1}}{F_{G2M} - F_{G1}} \tag{3}$$

where F^{lu} is the mean red fluorescence of BrdUrd-positive undivided cells (visually identified on the fluorescence plane), F_{G1} and F_{G2M} are the mean red fluorescences of $G1$ and $G2M$ cells, respectively. The relative movement defined by equation (3) is an increasing function of the time t after the end of the pulse and, in the case of a deterministic S phase, should reach the unity at $t = T_S$. The following estimates of T_S, based on a single DNA-BrdUrd histogram measured at time t, were proposed in (Begg *et al.*, 1985):

$$T_S^{(1)} = \frac{0.5\,t}{RM(t) - 0.5}, \qquad T_S^{(2)} = \frac{0.4\,t}{RM(t) - 0.6} \tag{4}$$

The first estimate was derived assuming a linear increase of RM starting from 0.5 at $t = 0$, whereas the second takes into account empirically the observed downward concavity in the time pattern of the relative movement.

White and Meistrich (1986) developed subsequently a theoretical analysis of the RM curve under the following assumptions: i) all S–phase cells are characterized by the same constant rate of DNA synthesis; ii) there is no variability of the transit time in $G2M$, T_{G2M}; iii) the population is in balanced exponential growth with no cell loss; iv) the red fluorescence is proportional to the DNA content; and v) the length of the BrdUrd pulse is infinitesimal. The expression obtained for $RM(t)$ depends on the kinetic parameters of the cycle in a rather complicated fashion (see next section). On the basis of a linear approximation of $RM(t)$ for $t > T_{G2M}$, the following estimate of T_S was proposed

$$T_S^{(3)} = \frac{0.5\,t}{RM(t) - RM_0}, \quad RM_0 = \frac{f_S - Z}{Z(f_S + f_{G2M})}, \quad Z = \ln\frac{1 + f_S + f_{G2M}}{1 + f_{G2M}} \tag{5}$$

where f_S and f_{G2M} denote the fraction of cells in S and $G2M$, respectively.

Having obtained an estimate of T_S, the potential doubling time of the population T_{pot}, i.e. the doubling time in the absence of cell loss, can be estimated by the formula

$T_{pot} = \lambda T_S / f_S$, where λ is a correction factor that depends on the growth fraction and the phase transit times (Steel, 1977). In (Wilson *et al.*, 1988; Riccardi *et al.*, 1988) the first of Begg's formulae (4) was applied to calculate T_S and the Steel's formula to calculate T_{pot} for *in vivo* human tumours.

The problem of determining λ and f_S (f_S is not easily found from a bivariate histogram measured some hours after the pulse) can be avoided by computing T_{pot} through the so-called ν function (White *et al.*, 1990a) as

$$T_{pot} = \ln 2 \frac{T_S}{\nu}, \qquad \nu = \ln \frac{1 + f^{lu}(t)}{1 - f^{ld}(t)/2} \tag{6}$$

where f^{lu} and f^{ld} denote, respectively, the fraction of labelled undivided cells and the fraction of cells generated by division of labelled cells. In balanced exponential growth (also in the presence of $G0$ cells) ν is constant for $T_{G2M} \leq t \leq T_S + T_{G2M}$ (White *et al.*, 1990a).

4. A Generalized Expression for $RM(t)$

The expression for $RM(t)$ given in White and Meistrich (1986) can be extended by allowing the rate of DNA synthesis $v(x)$ to change across S phase and by taking into account the finite length Δ of the BrdUrd pulse. The other assumptions ii), iii), and iv) of Section 3 still hold.

Let $n(x,t)$ be the cell density in S phase with respect to the DNA content x at time t, $n(x,t)\,\mathrm{d}x$ being the number of cells with DNA content between x and $x + \mathrm{d}x$; $n(x,t)$ satisfy the continuity equation (Bertuzzi *et al.*, 1983)

$$\frac{\partial}{\partial t} n(x,t) + \frac{\partial}{\partial x}[v(x)n(x,t)] = 0 \tag{7}$$

Now let t be the time after the end of the BrdUrd pulse at which the histogram is measured. A cell with DNA content x at time t has entered S phase at time $t - \phi(x)$, and thus is labelled if $t - \phi(x) < 0$, that is if $x > u(t)$. Thus, denoting by $n^l(x,t)$ the density of BrdUrd-labelled S–phase cells at the time t, we have for $0 \leq t \leq T_S$

$$n^l(x,t) = \begin{cases} 0 & \text{if} \quad 1 < x \leq u(t) \\ n(x,t) & \text{if} \quad u(t) < x < 2 \end{cases} \tag{8}$$

Let $N_2^l(t)$ be the number of BrdUrd-labeled $G2M$ cells at time t. According to equation (7), the number of cells per unit time entering $G2$ phase at time τ is given by $v(2)n(2,\tau)$, so that we have

$$N_2^l(t) = \begin{cases} \displaystyle\int_{-\Delta}^{t} v(2)n(2,\tau)\,\mathrm{d}\tau & \text{if} \quad 0 \leq t \leq T_{G2M} - \Delta \\ \displaystyle\int_{t-T_{G2M}}^{t} v(2)n(2,\tau)\,\mathrm{d}\tau & \text{if} \quad T_{G2M} - \Delta \leq t \leq T_S \end{cases} \tag{9}$$

The mean red fluorescence F^{lu} of the labelled undivided cells can be written as

$$F^{lu}(t) = \frac{\int_1^2 m(x)n^l(x,t)\,dx + m(2)N_2^l(t)}{\int_1^2 n^l(x,t)\,dx + N_2^l(t)} \tag{10}$$

where $m(x)$ is the mean red fluorescence of cells with DNA content x. From (10), assuming for $m(x)$ the expression $m(x) = F_{G1} + (F_{G2} - F_{G1})(x-1)$, the relative movement defined in equation (3) is given by

$$RM(t) = \frac{\int_1^2 (x-1)n^l(x,t)\,dx + N_2^l(t)}{\int_1^2 n^l(x,t)\,dx + N_2^l(t)} \tag{11}$$

where $n^l(x,t)$ and $N_2^l(t)$ are given by (8) and (9), respectively.

In balanced exponential growth there is $n(x,t) = N_0 e^{\alpha t} f(x)$, where N_0 is the number of cells at $t = 0$, α is the population growth rate constant, and $f(x)$ the cell frequency in S phase. The frequency function $f(x)$ can be expressed (Bertuzzi *et al.*, 1983) as

$$f(x) = \alpha(2 - f_{G1}) \exp[-\alpha\phi(x)]/v(x) \tag{12}$$

where f_{G1} is the $G1/G0$ phase fraction. Hence, equations (8) and (9) become

$$n^l(x,t) = \begin{cases} 0 & \text{if} \quad 1 < x \le u(t) \\ \alpha N_0(2 - f_{G1}) \exp[-\alpha(\phi(x) - t)]/v(x) & \text{if} \quad u(t) < x < 2 \end{cases} \tag{13}$$

and

$$N_2^l(t) = \begin{cases} N_0(2-f_{G1})\left[e^{-\alpha(T_S-t)} - e^{-\alpha(T_S+\Delta)}\right] & \text{if } 0 \le t \le T_{G2M} - \Delta \\ N_0(2-f_{G1})\left[e^{-\alpha(T_S-t)} - e^{-\alpha(T_S+T_{G2M}-t)}\right] & \text{if } T_{G2M} - \Delta \le t \le T_S \end{cases} \tag{14}$$

By substituting equations (13) and (14) into (11), after some rearrangement we obtain

$$RM(t) = \begin{cases} RM'(t) + \alpha \dfrac{\int_t^{T_S} [u(\tau) - 1 - \tau/T_S] e^{\alpha(t-\tau)}\,d\tau}{1 - e^{-\alpha(T_S+\Delta)}} & \text{if } 0 \le t \le T_{G2M} - \Delta \\ RM''(t) + \alpha \dfrac{\int_t^{T_S} [u(\tau) - 1 - \tau/T_S] e^{\alpha(t-\tau)}\,d\tau}{1 - e^{-\alpha(T_S+T_{G2M}-t)}} & \text{if } T_{G2M} - \Delta \le t \le T_S \end{cases} \tag{15}$$

where

$$RM'(t) = \frac{\alpha t + 1 - e^{-\alpha(T_S-t)} - \alpha T_S e^{-\alpha(T_S+\Delta)}}{\alpha T_S(1 - e^{-\alpha(T_S+\Delta)})} \tag{16}$$

$$RM''(t) = \frac{\alpha t + 1 - e^{-\alpha(T_S-t)} - \alpha T_S e^{-\alpha(T_S+T_{G2M}-t)}}{\alpha T_S(1 - e^{-\alpha(T_S+T_{G2M}-t)})} \tag{17}$$

We note that $RM'(t)$ and $RM''(t)$ give the relative movement when $v(x)$ is constant, and coincide with the expressions found in White and Meistrich (1986) if $\Delta = 0$; it is easy to see that the additional terms in (15) are positive (negative) if $v(x)$ is decreasing (increasing).

Figure 2 shows the behaviour of $RM(t)$ computed according to equation (15) for the following $v(x)$ functions, all with the same T_S ($T_S = 10\,\mathrm{h}$) : $v(x)$ constant; linearly increasing with $v(1) = 0.5v(2)$; linearly decreasing with $v(2) = 0.5v(1)$. As it can be seen in the figure, the $v(x)$ pattern can affect significantly the relative movement. This fact will cause an uncertainty of the estimates of T_S obtainable from formulae (4) and (5), which will be added to the uncertainty due to experimental errors. For instance, from the RM value at $t = 5\,\mathrm{h}$ of curve (a) we find $T_S{}^{(1)}$ and $T_S{}^{(3)}$ equal to 8.04 h and 9.51 h, respectively; from curve (b) we find 9.39 h and 11.47 h; and from curve (c) 7.10 h and 8.22 h.

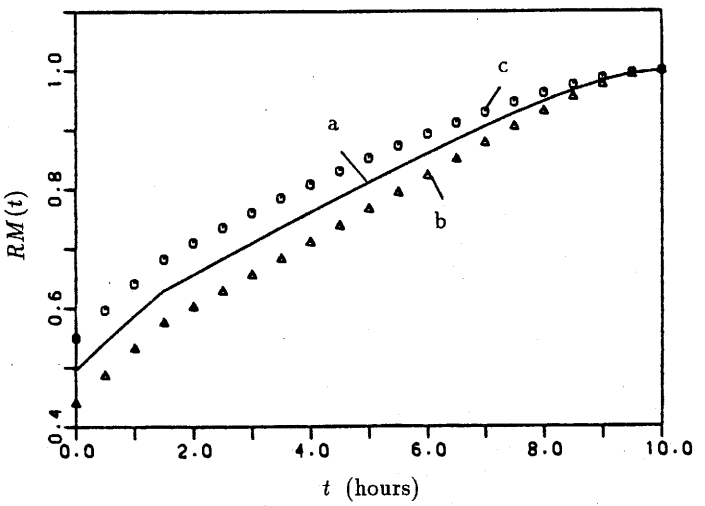

Fig. 2. Relative movement for $v(x)$ constant (a), linearly increasing (b), and linearly decreasing (c); $T_S = 10\,\mathrm{h}$, $T_{G2M} = 2\,\mathrm{h}$, $\alpha = 0.0315\,\mathrm{h}^{-1}$, $\Delta = 30$ min.

5. Sequences of DNA-BrdUrd Histograms

The availability of a sequence of DNA-BrdUrd histograms measured at increasing times after the end of the BrdUrd incorporation allows us to visualize the movement of the labelled cells across the cycle, giving thus easily a guess of cycle phase durations. The use of mathematical methods in conjunction with these data yields, for populations in balanced exponential growth, a more complete and accurate description of the cell cycle kinetics (Sasaki *et al.*, 1986; Yanagisawa *et al.*, 1985; White *et al.*, 1990b; Ubezio *et al.*, 1991).

From a sequence of histograms the $RM(t)$ curve can be monitored. Another interesting quantity that can be derived from the histograms is the depletion function,

DF, defined (White *et al.*, 1990b) as

$$DF = \ln(1 + f^{lu}) \tag{18}$$

For cells in balanced exponential growth (and assuming Δ negligible) the following expression for $DF(t)$ has been derived (White *et al.*, 1990b)

$$DF(t) = \begin{cases} \ln(1+e^{-\alpha t}[e^{\alpha(T_S+T_{G2M})} - e^{\alpha T_{G2M}}]) & \text{if} \quad 0 \le t \le T_{G2M} \\ \alpha(T_S + T_{G2M}) - \alpha t & \text{if} \quad T_{G2M} \le t \le T_S + T_{G2M} \end{cases} \tag{19}$$

In contrast with the result found for $RM(t)$, it is easy to show using (13) and (14) that equation (19) (or its extension for finite Δ) holds irrespective of the pattern of $v(x)$.

Estimates of α, T_S, and T_{G2M} were obtained for *in vitro* CHO cells by means of a fitting procedure of RM and/or DF data (White *et al.*, 1990b). When the rate of DNA synthesis across S phase appears to be non-constant, a more appropriate fitting of the data of relative movement should be based on expression (15). Note that in the absence of quiescent cells there is $\alpha = \ln 2/T_c$, with T_c the cycle time, so that the analysis based on the RM and DF data yields also an estimate of the $G1$ transit time.

Finally, White *et al.* (1991) stressed the interest of determining the relative movement of all the labelled cells (both undivided and divided) and of the unlabelled cells for a time length at least equal to $T_c + T_{G2M}$. For $v(x) =$ constant, the analytical expression of these relative movements depends on T_{G1}, T_S, T_{G2M}, and on the growth fraction of the population. Thus, estimates of these parameters can be obtained by optimal fitting of the experimental data.

6. Conclusions

From a bivariate DNA-BrdUrd distribution measured at the end of a pulse of short length, the mean BrdUrd-fluorescence across S phase gives with reasonable accuracy the profile of the rate of DNA synthesis, provided that the linearity conditions are fulfilled. If the pulse is not short enough, the profile of the mean BrdUrd-fluorescence is a deformed representation (with enhanced downward concavity) of the rate of DNA synthesis, the deformation being related to the actual values of the rate and to the length of the pulse.

The relative movement technique, based on the measurement of a single DNA-BrdUrd distribution some hours after the end of the pulse, allows a straightforward estimation of the DNA synthesis time T_S. However, a considerable error may affect this estimate, caused either by measurement errors and by model errors on $RM(t)$. We give a new analytical expression for the relative movement and, on this basis, we show that a source of error is related to the unknown shape of the rate of DNA synthesis across S phase.

An improved estimate of T_S and estimates of other kinetic parameters can be obtained if a sequence of DNA-BrdUrd histograms, measured at increasing times after the pulse, is available. From the relative movement computed along the sequence (or

from other quantities obtainable from the histograms), the cell kinetic parameters in balanced exponential growth may be estimated through optimal fitting of a suitable mathematical model. A promising method appears to be the simultaneous fitting of relative movement and of depletion function data.

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